Relative Binding Affinity-Serum Modified Access (RBA-SMA) Assay Predicts the Relative *In Vivo* Bioactivity of the Xenoestrogens Bisphenol A and Octylphenol

Susan C. Nagel,[1] Frederick S. vom Saal,[1] Kristina A. Thayer,[1] Minati G. Dhar,[1] Michael Boechler,[1] and Wade V. Welshons[2]

[1]Division of Biological Sciences; [2]Department of Veterinary Biomedical Sciences, University of Missouri-Columbia, Columbia, MO 65211 USA

We have developed a relative binding affinity-serum modified access (RBA-SMA) assay to determine the effect of serum on the access of xenoestrogens to estrogen receptors within intact cultured MCF-7 human breast cancer cells. We used this assay to predict low dose activity of two xenoestrogens in mice. In serum-free medium, bisphenol A, a component of polycarbonates and of resins used to line metal food cans, showed a lower relative binding affinity (RBA; 0.006%) than octylphenol (0.072%) and nonylphenol (0.026%), which are used as surfactants in many commercial products (all RBAs are relative to estradiol, which is equal to 100%). In 100% serum from adult men, bisphenol A showed a higher RBA (0.01%) than in serum-free medium and thus enhanced access to estrogen receptors relative to estradiol. In contrast, octylphenol showed a 22-fold decrease in RBA (0.0029%) and nonylphenol showed a 5-fold decrease in RBA (0.0039%) when measured in adult serum. This indicates that, relative to estradiol, serum had less of an inhibitory effect on the cell uptake and binding in MCF-7 cells of bisphenol A, while serum had a greater inhibitory effect on octylphenol and nonylphenol relative to estradiol. Extrapolation of these relative activities in adult serum predicted that the estrogenic bioactivity of bisphenol A would be over 500-fold greater than that of octylphenol in fetal mouse serum. Bisphenol A and octylphenol were fed to pregnant mice at 2 and 20 μg/kg/day. Exposure of male mouse fetuses to either dose of bisphenol A, but to neither dose of octylphenol, significantly increased their adult prostate weight relative to control males, which is consistent with the higher predicted bioactivity of bisphenol A than octylphenol in the RBA-SMA assay. In addition, our findings show for the first time that fetal exposure to environmentally relevant parts-per-billion (ppb) doses of bisphenol A, in the range currently being consumed by people, can alter the adult reproductive system in mice. *Key words*: bisphenol A, endocrine disruptors, environmental estrogens,17β-estradiol, fetus, 4-nonylphenol, 4-octylphenol, prostate, sexual differentiation, xenobiotics, xenoestrogens. *Environ Health Perspect* 105:70–76 (1997)

Recently, considerable attention has focused on a wide variety of endocrine disrupting chemicals in the environment that have estrogenic activity, variously referred to as environmental estrogens, xenobiotic estrogens, or xenoestrogens. Bisphenol A and the alkylphenols (nonylphenol and octylphenol) have been reported to have estrogenic activity (*1–5*). Bisphenol A is an essential component of epoxy resins used in the lacquer lining of metal food cans (*6*), as a component of polycarbonates (*3*), and in dental sealants (*7*). Octylphenol and nonylphenol are industrial additives used in a wide variety of detergents and plastics; these compounds are reported to be environmentally persistent (*5*). Bisphenol A, nonylphenol, and octylphenol are synthetic chemicals that are not estrogenic by design, but were only accidentally discovered to have estrogenic activity. Many other synthetic compounds have also been reported to show estrogenic activity (*8*). Development of rapid, inexpensive *in vitro* assays to accurately predict the estrogenic activity of man-made chemicals in humans and animals is thus of considerable importance with regard to public health and the preservation of wildlife.

A number of *in vitro* assays have been developed to characterize the activity of estrogens, and many of these assays are now in use to characterize the estrogenic activity of xenoestrogens in the environment. MCF-7 cell proliferation (*9–11*) or the E-SCREEN (*8,12*) is one of the most sensitive *in vitro* bioassays to measure estrogenic activity; concentrations in medium as low as 0.2 pM of 17β-estradiol (0.05 pg/ml; 0.05 parts per trillion) can produce a significant increase in proliferation relative to baseline (*13*). However, while the MCF-7 cell proliferation assay is excellent for detecting the estrogenic activity of known compounds or the presence of estrogenic activity in mixtures of unknown chemical composition, the cell proliferation assay may not be the most predictive *in vitro* assay for determining overall bioactivity of specific xenoestrogens in animals. One major reason is that the MCF-7 cell proliferation assay is conducted in tissue culture medium which contains only 5–10% serum and does not address how xenoestrogens are influenced by pharmacokinetics and by components of serum that dramatically influence the activity of steroidal estrogens, which are primarily bound to serum proteins in circulation (*14*).

The bioactivity of a xenoestrogen in the animal is affected by four key factors:

- Absorption and metabolism relative to the route of exposure
- Partitioning between aqueous and lipid compartments
- Effective concentration determined by how it is carried in circulation
- Intrinsic estrogenic activity of the molecule through its binding to and activation of estrogen receptors.

Currently, most *in vitro* assays measure primarily the fourth factor, intrinsic estrogenic activity of xenoestrogens. We have undertaken to develop *in vitro* assays to assess the additional factors, beginning with how xenoestrogens are carried in blood.

To determine whether the effects of serum on cell uptake can aid in estimating the bioactivity of xenoestrogens in animals and ultimately in humans, we developed the relative binding affinity-serum modified access (RBA-SMA) assay. In this assay, we examine how serum modifies the access of xenoestrogens to intracellular estrogen receptors within intact MCF-7 human breast cancer cells by comparing the relative binding affinity (RBA; relative to estradiol)

Address correspondence to W.V. Welshons, Department of Veterinary Biomedical Sciences, E102 Veterinary Medicine, University of Missouri-Columbia, Columbia, MO 65211 USA.

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of a xenoestrogen in serum-free medium to its RBA measured in 100% serum. Our *in vitro* assay takes into account the intrinsic activity of xenoestrogens (measured as affinity for the estrogen receptor), as do other *in vitro* assays. However, the RBA-SMA assay includes the effects of how xenoestrogens are carried in blood and the partitioning of xenoestrogens in serum and cell lipids by using 100% serum and live human cells rather than cell-free systems.

We examined the effect of serum on the estrogenic activity of the xenoestrogens bisphenol A, octylphenol, and nonylphenol in the RBA-SMA assay. The estrogenic activities of these compounds have been examined in other *in vitro* assays (*3,5*), with bisphenol A reported to be approximately 10-fold less estrogenic than octylphenol (*15*). However, in the RBA-SMA assay, bisphenol A was determined to be considerably more estrogenic than octylphenol, therefore we predicted that the relative potencies *in vivo* of bisphenol A and octylphenol would differ from those found by current *in vitro* methods. We administered bisphenol A and octylphenol to pregnant mice and examined whether this led to a permanent increase in prostate weight in the male offspring at 6 months of age, since we have previously described that this is a sensitive bioassay for detecting a very small increase in serum estradiol in mouse fetuses (*16,17*). Our prediction from the RBA-SMA assay that bisphenol A would alter fetal development in mice at a significantly lower dose than octylphenol was confirmed when the same doses of these compounds were fed to pregnant mice to compare their bioactivity in terms of effects on prostate enlargement in male offspring. In addition to demonstrating the utility of the RBA-SMA assay, a significant finding bearing on environmental xenoestrogens is that the doses of bisphenol A that affected fetal development were within the current range of human exposure to bisphenol A (*6,7*).

Materials and Methods

Materials. Minimum essential medium (MEM with nonessential amino acids, powdered), HEPES, bovine insulin, calf thymus DNA type I, Hoechst dye 33258, streptomycin sulfate, penicillin-G, 17β-estradiol (MW 272.4), EDTA, Hanks' balanced salt solution (HBSS), bovine serum albumin (BSA), and human male serum were obtained from Sigma Chemical Company (St. Louis, MO); all were "cell culture tested" when available. Bovine calf serum, phenol red (sodium salt), and lyophilized trypsin were obtained from Gibco BRL (Grand Island, NY). Bisphenol A (research grade, MW 228.3) was obtained from Aldrich Chemical Company (Milwaukee, WI). 4-Octylphenol (MW 208.3) technical grade and 4-nonylphenol (MW 222.35) technical grade were purchased from Chem Service (West Chester, Pennsylvania). 1,2,6,7[^{3}H]estradiol, 104 Ci/mol, was purchased from DuPont New England Nuclear (Boston, MA). All other chemicals were reagent grade.

Cell culture. MCF-7 cells were obtained from V. Craig Jordan, University of Wisconsin-Madison. MCF-7 cells were maintained in MEM with nonessential amino acids, 10 µg/ml phenol red, 10 mM HEPES, 6 ng/ml insulin, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% charcoal-stripped calf serum (maintenance medium) (*18,19*). Because the responsiveness of MCF-7 cells that are maintained continuously in stripped calf serum can drift (*20*), cells were propagated in stripped calf serum for approximately 1 year and then replaced with cells derived from our primary source—MCF-7 cells which had been maintained in whole serum before storage in liquid N_2.

Relative binding affinity assay. Relative binding affinity assays were performed in 24-well tissue culture plates. MCF-7 cells were seeded at approximately 100,000 cells per well in estrogen-free medium (maintenance medium without phenol red), and the cells were cultured for 3 days, with fresh medium added 1 day prior to the assay. Adult human serum was thawed, the pH was adjusted to 7.0, and the serum was filtered through a series of filters from 0.8 to 0.2 µm, including a Gelman G20 glass fiber prefilter (1.0 µm; Gelman Sciences, Ann Arbor, MI), to remove lipid precipitate.

RBA analyses were conducted as competition assays against 1 nM [^{3}H]estradiol in serum-free medium (SFM; MEM with nonessential amino acids plus 10 mM HEPES) or against 10 nM [^{3}H]estradiol in 100% serum. The concentration range of unlabeled test compounds was 10^{-7}–10^{-4} M in both SFM and 100% serum. The concentration range of nonradioactive estradiol (the reference competition) was 10^{-10}–10^{-7} M in SFM, and 10^{-9}–10^{-6} M in 100% serum. Test solutions were prepared in glass with 1% solvent ethanol present and then allowed to equilibrate with serum components for 1 hr at 37°C under 5% CO_2 prior to the assay. Cells were incubated with 0.5 ml/test media/well at 37°C and 5% CO_2 for 18 hr. At the end of the incubation, the medium was removed and the wells were washed three times with 1 ml HBSS that contained bovine serum albumin (BSA) at 2 mg/ml, once with 2 ml HBSS alone, and finally with 3 ml HBSS. The washed cells were dissolved (*21*) in 1 ml 10 mM EDTA, pH 12.4 (25°C, 15 min), neutralized (final pH ≈ 7.2) with 0.1 ml 0.77 M KH_2PO_4, and sonicated. Aliquots were then taken for scintillation counting or measurement of DNA.

Relative binding affinity calculation. For each xenoestrogen and for nonradioactive reference estradiol, the concentration required to inhibit 50% (IC_{50}) of specifically bound [^{3}H]estradiol was determined. To calculate the RBA, the IC_{50} for unlabeled reference estradiol was divided by the IC_{50} for each xenoestrogen, and this number was expressed as a percent. This same calculation was performed on data from the assay using 100% serum as the medium. This allowed us to determine whether serum changed the RBA of the xenoestrogen.

Relative binding affinity-serum modified access calculations. The RBA-SMA assay determines the effect of serum on the access of xenoestrogens to intracellular receptors within intact MCF-7 human breast cancer cells. The RBA value (relative to estradiol) obtained for the xenoestrogen in 100% human serum is divided by the RBA obtained in serum-free medium, and we termed this ratio the serum modified access (SMA) value. When an SMA value = 1 for binding to intracellular estrogen receptors, the xenoestrogen exhibited the same binding affinity relative to estradiol in the presence or absence of serum. That is, the xenoestrogen exhibited the same change in potency as did estradiol in the presence of serum. However, an SMA value >1 indicates that the xenoestrogen has greater access to intracellular estrogen receptors when reaching the cells from serum; conversely, an SMA <1 indicates that the presence of serum reduced the access of the xenoestrogen to intracellular estrogen receptors, again relative to estradiol.

DNA assay. DNA was measured fluorometrically in an aliquot of the sonicate using Hoechst dye 33258 according to the method of Labarca and Paigen (*22*). Calf thymus DNA was used as the standard after calibration by absorbance at 254 nm, assuming 20 absorbance units for 1 mg DNA per ml.

Animals, housing and mating. CF-1 mice (*Mus musculus domesticus*) were initially obtained in 1979 from Charles River Laboratories (Wilmington, MA) and were maintained on Purina Laboratory Chow (Richmond, IN) (5001) as an outbred colony in a closed facility since that time. Animals were housed in standard (11.5 × 7.5 × 5 in) polypropylene mouse cages on corn cob bedding. Rooms were maintained at 25°C ± 2° under a 12:12 light:dark (L:D) cycle, with lights on at 12 P.M. (so that timed mating could occur at the end of the dark phase, i.e., in mid- to late morning).

The males exposed to chemicals during fetal life were produced by mating adult virgin female mice. Females were time mated by being placed daily with a stud male for 4 hr at the end of the dark phase of the L:D cycle. Mating was verified by the presence of a seminal plug. Females were allowed to deliver normally on gestation day 19. Pups were weaned at 23 days of age, and male littermates were housed three per cage.

One male per litter was randomly selected for use in this study to control for litter effects (the remaining animals were used in other studies), and this selected male was individually housed for 1 month. When CF-1 males are housed in groups, a nonlinear dominance hierarchy is often observed in which there is one dominant male and the remaining males are subordinate (*23*); dominance status has marked effects on reproductive organs in CF-1 male mice. However, individual housing for 1 month eliminates the prior effects of subordination (F. vom Saal, unpublished observation).

Administration of xenoestrogens to pregnant females. Two different doses of bisphenol A and octylphenol were fed to pregnant mice (seven per group), at 2 and 20 µg/kg body weight (bw). Chemicals were dissolved in tocopherol-stripped corn oil (Cat# 901415; ICN, Aurora, OH), and 30 µl of oil containing different concentrations of each chemical was fed to pregnant mice one time per day at 10 A.M. from gestation day (GD) 11 to GD 17. This time in pregnancy was chosen to expose the fetuses to the xenoestrogens throughout the prenatal period of sexual differentiation and during the initial differentiation of the prostate (*24*). The last dose was administered on GD 17 to reduce the possibility of interfering with normal parturition; interference occurs with high doses of estrogenic chemicals (*25*). Bisphenol A, octylphenol, and control corn oil alone were fed with a micropipettor to the dams, who readily accept the oil without the potential stress of gavage. There were two control groups: one group (unhandled controls, n = 5) remained unhandled throughout pregnancy and the other group (vehicle controls, n = 6) was fed 30 µl of corn oil from GD 11 to GD 17.

Prostate collection. Body weight was determined for the randomly selected individually housed males at 6 months of age and then the males were killed by CO_2 asphyxiation and cervical dislocation. The entire reproductive tract was removed. The entire prostate, including the ventral and dorsolateral lobes (*26*), was stripped off of the urethra, and prostate weight was determined using a Mettler (Hightstown, NJ) AE-163 analytical balance.

Statistical analysis. Prostate weight for males from the different prenatal treatment groups was compared by analysis of covariance (ANCOVA), with body weight used as the covariate. ANCOVA provides an adjusted organ weight after correcting all groups to a common body weight. If the ANCOVA showed that body weight did not account for a significant component of the variance in organ weight, the data were then reanalyzed using analysis of variance (ANOVA), and the group means were presented without being adjusted for body weight. Planned comparisons were made using the LS means test. The confidence level for determining statistical significance was 5%. The Statistical Analysis System (SAS, Cary, NC) on the University of Missouri mainframe IBM computer (White Plains, NY) was used for these analyses.

Results

Relative binding affinity-serum modified access assay. In the RBA-SMA assay, unlabeled xenoestrogens competed with [^{3}H]estradiol for binding to estrogen receptors in intact MCF-7 cells. Competition of unlabeled xenoestrogens, which are full estrogen agonists, is proportional to their affinity for the estrogen receptor and thus their estrogenic activity. The assay was first conducted in serum-free medium and an RBA was calculated. The same assay was then conducted in 100% serum, and the RBA obtained was compared to the RBA measured in serum-free medium to determine how serum modified the access of the xenoestrogen to intracellular estrogen receptors. Xenoestrogen competition profiles from a single experiment conducted in serum-free medium and in 100% adult serum are presented in Figure 1. In serum-free medium (Fig. 1A), octylphenol exhibited an RBA that was approximately 10-fold greater than the RBA of bisphenol A. However, when the competing compounds were tested in 100% serum, the relationship of the competition profiles was reversed and the RBA of bisphenol A was greater than that of octylphenol (Fig. 1B).

A summary of three independent RBA-SMA assays of bisphenol A, nonylphenol, and octylphenol is presented in Table 1. The table includes the RBA measured in serum-free medium, the RBA measured in 100% adult serum, and the calculated SMA. The RBA of bisphenol A in serum was 1.7-fold higher than that measured in serum-free medium (Table 1, SMA = 1.7 for bisphenol A), indicating that its biological impact relative to estradiol would be underestimated by this factor in serum-free assays. In contrast, nonylphenol and octylphenol both showed an SMA <1

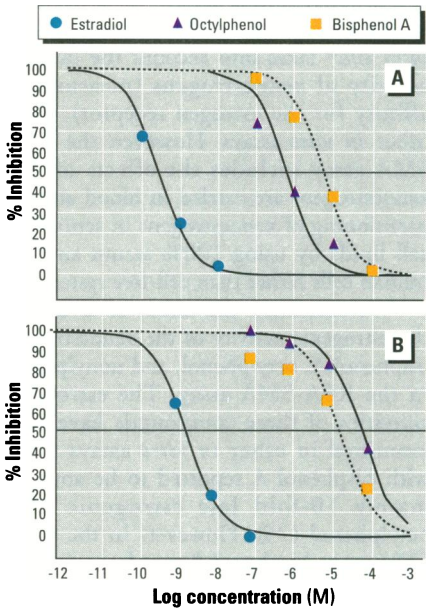

Figure 1. Relative binding affinity-serum modified access (RBA-SMA) assay. Unlabeled reference estradiol, octylphenol, or bisphenol A competed with (A) 1 nM [^{3}H]estradiol in serum-free media or (B) 10 nM [^{3}H]estradiol in 100% adult male serum. Specific binding averaged 3.8 fmol/µg DNA in SFM and 1.7 fmol/µg DNA in serum. Nonspecific binding ranged from 11 to 19% of total binding in SFM and 29 to 49% of total binding in serum.

(Table 1; SMA = 0.2 and 0.045, respectively), indicating that serum decreased their relative access to estrogen receptors by 5-fold and 22-fold, respectively. Hence, their activity relative to estradiol would be overestimated in serum-free assays. Clearly, the access of these three xenoestrogens was affected by the presence of serum, and the effect of serum on octylphenol and nonylphenol was in the opposite direction to its effects on bisphenol A. Because they showed the greatest difference in serum-modified access, bisphenol A and octylphenol were chosen to feed to pregnant mice to test the predictability of the RBA-SMA assay for bioactivity in the animal.

The predicted relative in vivo activity of bisphenol A and octylphenol in fetal mouse serum based on the RBA-SMA assay. The results obtained for the effects of serum on the activity of bisphenol A and octylphenol were used to estimate what the relative bioactivities would be in the animal, specifically in fetal mice where small increases in estradiol during development result in permanent developmental changes in the prostate (*17*) and increased prostate weight in adulthood (*16*). Ideally, the effects of serum-modified access on the estrogenic activity of these compounds would have been calculated directly from RBA values determined using fetal mouse serum.

Table 1. Relative binding affinity-serum modified access (RBA-SMA) assay

Compound	No. of assays	RBA in SFM (%) ± SE	RBA in serum (%) ± SE	SMA ± SE
Estradiol	3	100	100	1.00
Bisphenol A	3	0.0060 ± 0.0009	0.0100 ± 0.0012	1.70 ± 0.29
Nonylphenol	3	0.026 ± 0.0069	0.0039 ± 0.0011	0.20 ± 0.11
Octylphenol	3	0.072 ± 0.0152	0.0029 ± 0.0005	0.045 ± 0.013

Abbreviations: RBA, relative binding affinity; SFM, serum-free medium; SE, standard error; SMA, serum modified access (RBA in serum ÷ RBA in serum-free medium).
RBA analysis was conducted in SFM and in 100% adult male serum. SMA values are the mean of three independent RBA-SMA assays. Bisphenol A showed enhanced access from serum while octylphenol and nonylphenol showed decreased access from serum relative to estradiol. Average concentration that inhibited 50% (IC_{50}) values in serum-free medium were 5.64×10^{-10} M, 8.57×10^{-6} M, 2.74×10^{-6} M, and 6.30×10^{-7} M for estradiol, bisphenol A, nonylphenol, and octylphenol, respectively. Corresponding values in serum were 3.96×10^{-9} M, 3.94×10^{-5} M, 1.11×10^{-4} M, and 1.45×10^{-4} M, respectively.

Table 2. Predicted serum modified access (SMA)[a] in fetal mouse serum estimated by extrapolation from SMA in human serum

	Serum-free (100% free E_2)	Adult human (4% free E_2)	Estimated fetal mouse (0.2% free E_2)		Predicted SMA in fetal mouse
Bisphenol A	1.0	1.7	$(1.7 - 1) \times 20^b + 1$	=	15
Octylphenol	1.0	0.045	$0.045 \div 20^b$	=	0.0022

Abbreviations: E_2, 17β-estradiol; RBA, relative binding affinity.
[a]SMA = RBA in serum ÷ RBA in serum-free medium.
[b]4% ÷ 0.2% = 20-fold.

Table 3. Predicted estrogenic activity[a] calculated from the predicted serum modified access (SMA) for bisphenol A and octylphenol

	Predicted SMA in fetal mouse		RBA in serum-free medium		Predicted bioactivity[b]	Relative activity[c]
Bisphenol A	15	×	0.006%	=	0.09%	563
Octylphenol	0.0022	×	0.072%	=	0.00016%	1

RBA, relative binding affinity.
[a]Predicted SMA × RBA in serum-free medium = predicted RBA in fetal mouse serum.
[b]Predicted estrongenic activity in fetal mouse serum.
[c]Predicted bioactivity of bisphenol A/octylphenol.

However, the large volume of fetal mouse serum required for these measurements was not available. In the absence of fetal serum data, we extrapolated the SMA calculations obtained in adult male serum to what might be expected in fetal mouse serum by assuming that the effects on the SMA would be roughly proportional to the free fraction of estradiol in the two sera.

The free fraction of estradiol in fetal mouse serum during the time of sexual differentiation and initiation of prostate development is 0.2% (vom Saal et al., unpublished data), which is similar to that previously reported in fetal rats (*27*). The 0.2% free fraction of estradiol in fetal mice is 20-fold lower than the 4% free estradiol in the adult male serum (*28*) that we used to measure the SMA above (4% ÷ 0.2% = 20-fold). We have observed a linear relationship between the percent of free estradiol in serum and the SMA when the RBA assay was conducted with serial dilutions of adult serum. Therefore, we estimated that the effects of fetal mouse serum on the access of xenoestrogens to estrogen receptors would be approximately 20-fold greater than the effects we measured in adult serum. The deviations in the SMA from the value of 1.0 in adult serum were thus altered by a factor of 20 for both bisphenol A and octylphenol in order to predict the SMA in fetal serum; this calculation for conversion of the SMA in adult serum (4% free estradiol) to the SMA in fetal serum (0.2% free estradiol) is shown in Table 2. The calculation step (multiplication or division) involving the correction factor (20-fold) depends on whether the SMA value showed enhancement (SMA >1) or inhibition (SMA <1) of cell uptake. If the SMA >1, the degree of enhancement of cell uptake is determined by multiplying the SMA in adult serum by the correction factor; hence, the enhancement will be greater relative to estradiol. If the SMA <1, the assumption is that in fetal serum the cell uptake would be even less than estradiol, and the SMA value is divided by the correction factor.

The SMA values for bisphenol A and octylphenol predicted for fetal mouse serum were then applied to adjust the activity of the compounds measured in serum-free medium to the bioactivity predicted in fetal mouse serum (Table 3). The predicted bioactivity in fetal mouse serum was calculated by multiplying the RBA measured in serum-free medium by the predicted SMA in fetal serum. These calculations predicted that in the presence of fetal mouse serum (all other factors being equal), bisphenol A would exhibit over 500-fold greater bioactivity than would octylphenol in mouse fetuses, despite the higher intrinsic estrogenic activity of octylphenol that was observed before taking serum into account.

Effect of exposure to bisphenol A and octylphenol during fetal life on adult prostate weight in mice. We administered 2 and 20 µg/kg bisphenol A per day to pregnant mice; these doses are within the range of daily human exposure (*6,7*). Our expectation was that if the RBA-SMA assay correctly predicted estrogenic bioactivity in the animal, then the 20 µg/kg dose of bisphenol A would result in enlargement of the prostate in adult male offspring, similar to our earlier finding that a small increase in fetal serum estradiol permanently increased prostate size in mice (*16*). In contrast, we expected that the 2 µg/kg dose of bisphenol A might be too low to produce a detectable change in prostate weight. We predicted that feeding the same two doses of octylphenol would not result in a significant change in the prostate of male offspring because the RBA-SMA assay predicted that octylphenol would be 500-fold less active than bisphenol A (Table 2). This was a preliminary experiment to examine the predicted relative activities of bisphenol A and octylphenol from the RBA-SMA assay.

There were two control groups: one group (unhandled controls) remained unhandled throughout pregnancy, and the other group (vehicle controls) was fed 30 µl of corn oil from GD 11 to GD 17. There were no significant differences between the oil-fed controls and the unhandled controls in body weight or prostate weight, indicating that the method of administration did not affect these parameters. Therefore, these two groups were pooled and treated as one control group for the following analyses.

ANCOVA showed that prostate weight varied significantly as a function of prenatal treatment [$F(4,27) = 10.0$; $p<0.001$], while body weight did not account for a significant component of the variance in prostate weight. Based on these findings we reanalyzed prostate weight using ANOVA and again there was a significant effect of prenatal treatment on prostate weight ($p<0.01$). Both ANOVA and ANCOVA yielded identical results with regard to group means

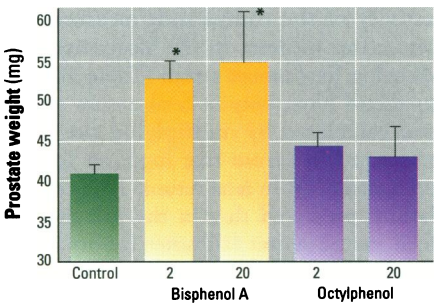

Figure 2. Prostate weight in adult male mice (6 months old) exposed as fetuses to bisphenol A or octylphenol. Mothers were fed 2 or 20 µg/kg body weight/day bisphenol A or octylphenol from days 11 to 17 of pregnancy. Error bars are the standard error; $n = 7$ for bisphenol A and octylphenol, and $n = 11$ for unexposed controls.
*$p<0.01$.

and group differences in prostate weight, and prostate weight in Figure 2 is thus presented uncorrected for body weight.

Prostates collected from 6-month-old males whose mothers had been fed bisphenol A at either 2 or 20 µg/kg bw/day were significantly heavier than prostates from control males (Fig. 2), weighing 30% and 35% more than control prostates at the low and high dose of bisphenol A, respectively. However, prostates from males exposed prenatally to either the 2 or 20 µg dose of octylphenol were not significantly different from the prostates from control animals (Fig. 2). Based on the doses that we administered in this preliminary experiment, bisphenol A appeared to be at least 100-fold more estrogenic than octylphenol, which is consistent with the prediction derived from the calculations from the RBA-SMA assay above. An interesting finding was that the coefficient of variation (CV) for prostate weight of animals exposed to the highest dose (20 µg) of both bisphenol A and octylphenol was three-fold greater than the CV for the animals exposed to the lower doses (2 µg), suggesting greater variance in response to the higher of these two low doses.

Body weight differed significantly as a function of prenatal treatment group [$F(4,27) = 3.8$; $p<0.05$). Males exposed to both the low dose of bisphenol A [34.6 ± 1.12; $n = 7$; mean ± standard error (SE) and octylphenol (33.4 ± 1.11, $n = 7$) were significantly lighter than control males (37.9 ± 0.83; $n = 11$), while exposure to the high doses of both bisphenol A and octylphenol (36.7 ± 1.12; $n = 7$ and 37.3 ± 0.73; $n = 7$, respectively) did not influence body weight. However, as described above, adjusting prostate weight for body weight did not alter the results with regard to group differences in prostate weight, since body weight was not correlated with prostate weight (Pearson's $r(39) = 0.19$; $p = 0.25$).

Discussion

In prior published assays of estrogenic activity that have not taken the effects of serum into account, bisphenol A was reported to have one-half to one-tenth of the estrogenic activity of octylphenol (*3,5,15*); when measured in serum-free medium in our RBA-SMA assay, bisphenol A showed one-tenth the activity of octylphenol and also exhibited a lower RBA than nonylphenol. However, in the portion of the RBA-SMA assay conducted in 100% adult human male serum, bisphenol A showed a greater relative estrogenic activity than either octylphenol or nonylphenol. The results of the RBA-SMA assay led to the prediction that bisphenol A would be substantially more estrogenic than octylphenol in the mouse fetus. This was confirmed when bisphenol A at maternal doses of 2 and 20 µg/kg stimulated prostate enlargement in male offspring, while octylphenol at the same doses did not alter prostate weight. The RBA-SMA assay therefore provided a substantial improvement over existing *in vitro* assays for predicting the bioactivity of these xenoestrogens in the animal.

The effects of serum on the cell uptake and binding to estrogen receptors of bisphenol A, nonylphenol, and octylphenol were in opposite directions. The activity of bisphenol A was slightly enhanced in serum while the activity of nonylphenol and octylphenol was dramatically decreased relative to estradiol. It is likely that there are multiple mechanisms mediating both the increased (SMA >1) and decreased (SMA <1) activity of xenoestrogens observed in serum relative to serum-free medium. While estradiol is primarily bound to proteins in serum, xenoestrogens that do not bind to these proteins may escape this mechanism, which limits the cell uptake of natural estrogens. Several xenoestrogens have been reported to show less binding to serum proteins than estradiol (*29–34*). This may increase their effective concentration relative to estradiol and lead to increased estrogenic activity of these compounds. Conversely, xenoestrogens that show greater binding to serum proteins or greater lipid partitioning may have a lower free fraction than estradiol, thus decreasing their effective concentration in serum. Octylphenol has been reported to show less binding to human serum proteins [both sex hormone-binding globulin (SHBG) and albumin] than estradiol (*34*); however, in the RBA-SMA assay, serum inhibited octylphenol from binding to the estrogen receptor more than estradiol. This suggests that factors other than binding to serum proteins, such as lipid partitioning (either serum or mammalian cell membrane), may play a role in the activity of some xenoestrogens; the alkyl chain present in octylphenol and nonylphenol (but not bisphenol A) may be important in this regard. However, at this time the mechanism of decreased access of xenoestrogens from serum is unknown and will require further study.

Most xenoestrogens are not available in radiolabeled form, so the effects of serum on their access to estrogen receptors could not be measured directly. Therefore, we used RBA analysis to compare the access of xenoestrogens with tritiated estradiol. The RBA-SMA assay measured access of xenoestrogens from human serum into live human cells. Ideally, the RBA-SMA assay should be conducted in serum from the animal and lifestage at which the estrogenic activity is in question. Because the quantities of fetal mouse serum required for the RBA-SMA assay were not available, we extrapolated the results from adult human to fetal mouse serum using the free fraction of estradiol as a reference (Tables 2,3); we predicted that bisphenol A would be over 500-fold more estrogenic than octylphenol in the mouse fetus. These two xenoestrogens were chosen for further study because they showed the greatest change in access to estrogen receptors in MCF-7 cells (the largest and smallest SMA values) between serum-free medium and serum.

For our *in vivo* bioassay, we focused on the potential for bisphenol A and octylphenol to exert estrogenic effects in the mouse fetus based on prior information on the potency of estradiol in fetal mice from studies of the intrauterine position (IUP) phenomenon. We have shown that a small increase in serum estradiol during fetal life was related to enlargement of the prostate in adulthood. Specifically, a 23-pg/ml (23 parts per trillion, ppt) increase in total serum estradiol in male mouse fetuses positioned *in utero* between two female fetuses (2F males; 101 pg estradiol/ml serum) relative to males between two male fetuses (2M males; 78 pg estradiol/ml serum) (*35*) was associated with a 20% increase in prostate weight in 2F males relative to 2M males in adulthood (*16*). Subsequently, we experimentally verified these findings in mice by implanting Silastic capsules (Dow Chemical, Midland, IN) containing estradiol into pregnant mice to produce a very small increase in total serum estradiol during reproductive tract organogenesis in male fetuses, which led to a 25–30% increase in prostate weight in treated males when they were adults (*17*).

These observations and the results of the RBA-SMA assay were used to calculate the circulating concentration or reference dose in pregnant mice that we expected to produce an increase in prostate weight in male offspring. Calculation of reference dose was

based on the IUP studies in which an increase in total serum estradiol of 23 pg/ml resulted in permanent prostate enlargement in 2F male mice relative to 2M male mice and on the predicted bioactivity of bisphenol A relative to estradiol in fetal mice from Table 3 (0.09%). With these two pieces of information, reference doses of bisphenol A and octylphenol were calculated that would be equivalent to an increase in serum estradiol in male fetuses of 23 pg/ml and would thus produce prostate enlargement. For comparison, reference doses were also calculated using the RBAs that we determined in serum-free medium; these relative activities are representative of the values that have been obtained with assays which do not take the effects of serum into account when measuring xenoestrogen activity (*3–5,15*).

This calculation (Table 4) predicted that bisphenol A at 21 µg/kg would lead to an increase in prostate weight in male mice; however, since octylphenol was predicted to be over 500-fold less estrogenic than bisphenol A, neither of the two doses we administered (2 and 20 µg/kg bw) was predicted to produce an increase in prostate weight in males exposed to octylphenol. When bisphenol A was administered by feeding to pregnant mice at 20 µg/kg, the approximate reference dose, there was a significant 35% increase in prostate weight in male offspring. In contrast, neither dose of octylphenol altered prostate weight.

Because the calculation of reference dose above does not include the effects of metabolism and pharmacokinetics and because bisphenol A also exhibited estrogenic effects at the lowest dose we administered (2 µg/kg), bisphenol A appeared to be more estrogenic *in vivo* than predicted. We (*17*) have found that the estrogenic activity of bisphenol A was only 100-fold lower (or less) than that of diethylstilbestrol (DES) when using the prostate as a bioassay, again indicating that bisphenol A appeared to be more estrogenic *in vivo* than predicted. Several possible mechanisms could explain this. Arnold et al. (*36*) reported that some xenoestrogens act synergistically and have greater activity in mixtures than predicted when the individual compounds are assayed alone. It is possible that bisphenol A acts synergistically with the phytoestrogens, including genistein, known to be present in soy-based mouse chow. Alternatively, bisphenol A may be bioactivated *in vivo* to a more estrogenic metabolite (in addition, octylphenol may be metabolized to a less estrogenic compound). Additionally, bisphenol A may bind even less to the serum proteins in fetal mouse serum than to those in adult human serum, thus having greater activity and greater SMA in fetal mouse serum than was predicted by extrapolation from results obtained in adult human serum. Finally, a novel estrogen receptor, termed ER-beta, has recently been cloned (*37*). ER-beta is expressed in the rodent prostate (*37*), and this estrogen receptor may have a different responsiveness for bisphenol A than does the classical ER-alpha present in MCF-7 cells.

Table 4. Calculation of xenoestrogen reference doses

Compound	Estradiol reference[a]		Predicted bioactivity[b]		Equivalent estrogenic activity[c]		MW, µg/µmol		Reference dose[d]
In serum									
Bisphenol A	84 pM	÷	0.09%	=	0.093 µM	×	228.3	=	21 µg/kg
Octylphenol	84 pM	÷	0.00016%	=	52.5 µM	×	208.3	=	10,900 µg/kg
In serum-free									
Bisphenol A	84 pM	÷	0.006%	=	1.400 µM	×	228.3	=	320 µg/kg
Octylphenol	84 pM	÷	0.072%	=	0.117 µM	×	208.3	=	24 µg/kg

[a]Fetal estradiol elevation that resulted in adult prostate enlargement: 84 pM (= 23 pg/ml) (*16,35*).
[b]From Table 3, activity relative to estradiol, based on relative molar concentrations; the predicted bioactivities SERUM-FREE are the RBAs measured in Serum-Free Medium.
[c]Molar concentration of xenoestrogen predicted to equal the estrogenic activity of the estradiol reference.
[d]Equivalent estrogenic activity in mol/liter converted to mass, µg/liter; value then expressed as µg/kg for reference dose; MW of bisphenol A = 228.3; MW of octylphenol = 208.3.

The RBA-SMA assay accurately predicted the relative bioactivity of bisphenol A and octylphenol in the animal by incorporating the effects of serum, whereas the intrinsic activity of the xenoestrogens measured in serum-free assays did not predict *in vivo* bioactivity (Table 4). The RBA-SMA assay is thus an improvement on existing *in vitro* assays for predicting the bioactivity of xenoestrogens in the animal. However, the inclusion of additional information, such as metabolism and pharmacokinetics of xenoestrogens, would further increase the accuracy of *in vitro* assays as predictors of bioactivity.

Xenoestrogens are often referred to as weak estrogens that are not likely to be biologically active at environmentally relevant concentrations. In our study, a maternal dose of only 2 µg/kg/day bisphenol A enlarged the prostate in male offspring. This dose is equivalent to a daily dose of 50 µg for a 25-kg child or 150 µg for a 75-kg adult. In this regard, Olea et al. (*7*) have shown that after a 50-mg dental sealant treatment, the saliva measured in a 1-hr collection after the application of the sealant from human subjects contained from 90 to 931 µg of bisphenol A. They also measured bisphenol A in the saliva of an individual who had tooth sealant applied 2 years earlier and found 66.4 µg in a 1-hr saliva collection before additional sealant treatment, suggesting that bisphenol A may be continually released after the initial dental work. Brotons et al. (*6*) analyzed vegetables packaged in food cans with lacquer coating and found that as much as 23 µg of bisphenol A was recovered from 50 ml of the liquid portion of one food can. An important aspect of our study is the proximity of the maternal doses of bisphenol A administered to pregnant mice and the reported ranges of human exposure to this chemical.

Although we did not find estrogenic effects of octylphenol in this animal study, Sharpe et al. (*38*) recently reported effects on the male reproductive tract in rats that were exposed to octylphenol administered orally to their mothers before pregnancy, during gestation, and throughout lactation. These effects were seen at doses that may have been at or above our highest dose, although it was difficult to determine postnatal dose, and prenatal dose was not determined (*38*). It is possible that if we administered these compounds throughout pregnancy and lactation, rather than just 7 days during pregnancy, there may have been a different outcome with regard to octylphenol. It is well known that different effects are seen with prenatal versus postnatal estrogen exposure in rodents (*35*). Also important here are the high levels of estrogens during fetal development versus the very low levels between birth and puberty (*27,39*), and this may also affect the sensitivity of the developing male reproductive tract to environmental estrogens.

If a xenoestrogen interacts with serum-binding proteins in the same manner as estradiol, then the RBA-SMA assay will not change the prediction of its estrogenic activity relative to estradiol. However, of the more than 12 xenoestrogens that we have tested thus far, none has shown the same serum-modified access as estradiol. By incorporating the effects of serum on xenoestrogens, the 2-day RBA-SMA assay provided an accurate prediction of the bioactivities of bisphenol A and octylphenol. This assay should thus be of considerable interest to toxicologists seeking to estimate the dose of a xenoestrogen to administer to animals within a physiological range of estrogenic activity rather than in the toxicological range used in traditional high-dose studies of toxicants. For systemic toxicants, the mechanism of action

is typically unknown, and a comparison of administered dose to the concentration of an endogenous standard (such as estradiol for xenoestrogens) cannot be made. In contrast, for a xenoestrogen, the RBA-SMA assay coupled with information about endogenous concentrations and the developmental effects of estrogen can be used to predict bioactive doses. Our findings, in addition, reveal that fetal exposure to bisphenol A can alter the reproductive tract in rodents at much lower doses than previously thought to be active. The predicted NOEL (no observed effect level) for bisphenol A has been estimated at 50 mg/kg bw/day (*40*); however, bisphenol A was active in rodents at 2 and 20 µg/kg/day, which lies near or within the reported ranges of current human exposure to this chemical (*6,7,40*).

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